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Induction of microthrombotic thrombocytopenia in normal mice by transferring a platelet-reactive, monoclonal anti-gp70 autoantibody established from MRL/*lpr* mice: an autoimmune model of thrombotic thrombocytopenic purpura

K. HASHIMOTO*†, N. TABATA*, R. FUJISAWA‡, H. MATSUMURA* & M. MIYAZAWA*‡
*Department of Immunology and †Third Department of Internal Medicine, Kinki University School of Medicine, Osaka, and ‡Department of Pathology, Tohoku University School of Medicine, Sendai, Japan

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SUMMARY

MRL/MpJ-*lpr/lpr* (MRL/*lpr*) mice spontaneously develop immune complex-mediated glomerulonephritis and thrombocytopenia. Although the presence of cross-reactive anti-phospholipid antibodies in sera of MRL/*lpr* mice has been demonstrated, possible relationships between detected autoantibodies and the development of thrombocytopenia have not been elucidated. Recent genetic analyses in a few different strains of lupus-prone mice have pointed out a close correlation between autoantibodies reactive with endogenous retroviral *env* gene product, gp70, and the development and severity of glomerulonephritis. In the process of establishing possibly nephritogenic anti-gp70 autoantibody-producing hybridoma cells from MRL/*lpr* mice, we identified an IgG2a-producing anti-gp70 hybridoma clone that induced microvascular intraluminal platelet aggregation, thrombocytopenia, and amenia upon transplantation into syngeneic non-autoimmune mice. This and two other anti-gp70 antibodies bound onto the surface of mouse platelets, and purified IgG2a of the anti-gp70 autoantibody induced glomerular lesions with characteristics of thrombotic thrombocytopenic purpura when injected into non-autoimmune mice. The pathogenic anti-gp70 autoantibody specifically precipitated a platelet protein with an approximate relative molecular mass of 40 000.

Keywords gp70 MRL/*lpr* platelet autoantibody thrombocytopenia

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a form of microangiopathic haemolytic anaemia and thrombocytopenia associated with platelet aggregation within the microcirculation [1–3]. Many different causative agents are proposed for this potentially fatal disease. These include bacterial toxins, viruses, autoantibodies, immune complexes, and certain drugs [2,3]. However, pathogenic mechanisms that induce characteristic intraluminal platelet thrombi with little or no involvement of fibrin are still unknown. The possibility that immunological events are involved in TTP is supported by studies that have demonstrated the presence in patients' plasma of IgG antibodies reactive with a platelet and/or endothelial cell antigen, especially CD36 [4,5]. In recent studies, IgG antibodies that inhibit von Willebrand factor (vWF)-cleaving protease were also found in patients with acute TTP [6,7]. In addition, a variety of immunological disorders, including systemic lupus erythematosus (SLE), rheumatoid arthritis, polymyositis and Sjögren's syndrome, have been found to be associated with TTP [8–11]. Although the causative relationship between these autoimmune conditions and TTP is largely unknown, there might be certain autoimmune mechanisms that are shared between systemic autoimmune diseases and TTP.

Spontaneous animal models are useful in determining the characteristics of autoantibodies that are required for their pathogenicity, and also in analysing the origins and mechanisms of autoantibody production. If there is an autoimmune mechanism shared between systemic autoimmune diseases and TTP, it might be delineated in animal models of autoimmunity. Male F_1 offspring of New Zealand white (NZW) and BXSB/Mp mice, $(NZW \times BXSB)F_1$, were first reported to have an abnormally high incidence of coronary vascular disease and myocardial

R.F. present address: Department of Microbiology, Kinki University School of Medicine, Osaka-Sayama, Osaka, Japan.

Correspondence: Professor Masaaki Miyazawa, Department of Immunology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan.

E-mail: masaaki@med.kindai.ac.jp

infarction [12]. The vascular lesion was not inflammatory and was associated with thrombosis of small arteries. They were later found to develop platelet-associated and circulating anti-platelet autoantibodies and progressive thrombocytopenia, in addition to lupus nephritis and the above-described degenerative coronary vascular disease [13], and have been used as a model of idiopathic (or autoimmune) thrombocytopenic purpura (ITP). Several clones of anti-platelet autoantibody-producing hybridomas were established from (NZW × BXSB)F_1 mice, and two of them have been shown to be pathogenic by transferring into nude mice [14]. In addition, the presence of anti-phospholipid autoantibodies in sera of (NZW × BXSB)F_1 mice was demonstrated, and monoclonal anti-phospholipid autoantibodies were established [15]. However, the exact molecular nature of the platelet antigens that were recognized by the above pathogenic clones of anti-platelet autoantibodies has not been identified, except for the molecular weight of yet undefined candidate antigens determined by Western blotting [14]. In addition, none of the monoclonal anti-phospholipid antibodies established from (NZW × BXSB)F_1 mice has been tested for their possible pathogenicity.

MRL/Mp mice homozygous for the Fas mutant *lpr* gene (MRL/*lpr*) are well-analysed mouse models of lupus nephritis, and they are also reported to develop thrombocytopenia and anti-phospholipid autoantibodies [16,17]. In recent genetic analyses [18,19] as well as in previous pathogenic studies [20], the product of an endogenous retrovirus *env* gene, gp70, expressed in the liver as a normal constituent of mouse serum [21], has been implicated in the pathogenesis of murine lupus nephritis as the major component of nephritogenic immune complexes. In the process of establishing monoclonal anti-gp70 autoantibodies from MRL/*lpr* mice and examining their possible pathogenicity, we found an IgG2a-producing anti-gp70 hybridoma clone that induced acute haemorrhagic death upon transplantation into syngeneic non-autoimmune (BALB/c × MRL/Mp-+/+)F_1 mice. Histopathologic and electron microscopic analyses revealed diffuse intraluminal platelet aggregation in the transplanted mice. We report here that the anti-gp70 autoantibody directly binds onto the surface of mouse platelets, and it can induce thrombotic microangiopathy with some characteristics of TTP when injected into syngeneic non-autoimmune mice.

MATERIALS AND METHODS

Mice

Breeding pairs of MRL/MpJ-+/+(MRL/+) and MRL/*lpr* mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). These strains of mice were maintained by sister–brother mating in our animal facilities under specific pathogen-free conditions. BALB/cCrSlc mice were also purchased from Japan SLC, and (BALB/c × MRL/+)F_1 hybrid mice were bred in our animal facilities. All the animal experiments described in this study were approved by the institutions, and performed under the guidelines of our animal facilities.

NZB xenotropic virus-producing cells

NZB-AR cells that are chronically infected with a biological clone of NZB xenotropic virus were kindly provided by Dr L. Evans (Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases, Hamilton, MT). Control uninfected Mv1Lu mink lung cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Expression of the murine leukaemia viral env *gene in a recombinant vaccinia virus*

A vaccinia virus transfer vector used for the expression of the mouse retrovirus *env* gene was constructed as described previously [22–24]. Plasmid clone $pNZB_{9-1}$ [25] containing the whole permuted infectious molecular clone of an NZB xenotropic virus, IU-6, was used as the source of endogenous xenotropic virus *env* gene sequence. The *Hin*cII-*Sma*I fragment harbouring the entire *env* gene and portions of the *pol* and LTR from $pNZB_{9-1}$ was reconstructed in pBluescript-KS(+) vector from purified *Hin*cII-*Eco*RI and *Eco*RI-*Sma*I fragments, and the unique *Acc*I site upstream of the *env* initiation codon was replaced with a *Bam*HI linker. A *Bam*HI-digested fragment containing the entire *env* gene and a part of the LTR was subcloned into the unique *Bgl*II site of the previously described modified vaccinia virus transfer vector pSC11-SB [22] to generate a vaccinia virus-NZB xenotropic virus *env* gene recombinant. Recombinant vaccinia viruses were produced by homologous recombination as described [22–24]. A recombinant vaccinia virus expressing the influenza virus haemagglutinin (HA) gene [26] was used as a negative control throughout the experiment.

Production and screening of hybridoma cells

Spleen and lymph node cells were prepared aseptically from unmanipulated MRL/*lpr* mice. P3/NSI/1-Ag4-1 (NS-1) myeloma cells were purchased from the ATCC and used as fusion partner cells. Hybridoma cell fusion, hypoxanthine-aminopterin-thymidine selection, and cloning by colony formation in fibrin gels were performed as described previously [27,28]. For immunofluorescence detection of the reactivities of hybridoma-derived antibodies to expressed *env* gene products, monkey CV-1 cells were grown in wells of 96-well tissue culture plates, infected with a recombinant vaccinia virus at 100–200 plaque-forming units (PFU) per well for 20–36 h, and incubated at 4°C overnight with a hybridoma culture supernatant added at 100 μl/well. After incubation, culture supernates were aspirated and the wells were washed twice with phosphate-buffered balanced salt solution (PBBS) [29] containing 2% fetal calf serum (FCS), and once with PBBS not containing FCS. Cells in each well were fixed with methanol, blocked with 10% skim milk, and were stained with a 1:150 dilution of FITC-conjugated goat anti-mouse immunoglobulin antibody (Cappel Organon Teknika Corp., West Chester, PA) as described [22]. For observation the plates were placed upside down under an Axioplan fluorescence microscope (Zeiss, Overkochen, Germany).

Hybridoma cells producing reference MoAbs that react with various mouse retrovirus *env* gene products [30–32] were kindly provided by Dr B. Chesebro (Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases). A hybridoma cell line, VL9G6, producing mouse IgG2a that reacts with the extracellular regions of human and rabbit very low density lipoprotein receptor (VLDLR), was established by immunizing (BALB/c × MRL/+)F_1 mice with a synthetic peptide, SLEQCGRQPVIHTK (human VLDLR amino acids 198–211), coupled with keyhole limpet haemocyanin, and screening the antibody-producing cells by peptide-specific ELISAs as described previously [33]. Specificity of this hybridoma antibody has been confirmed by immunofluorescence and Western blotting using the previously described transfectant [33]. Immunoglobulin isotypes of MoAb were determined by an Ouchterlony immunodiffusion method using an isotype-specific antibody kit (The Binding Site, Birmingham, UK) as described previously [27,28].

Bleeding and blood cell counts

Mice were anaesthetized with ether and bled from the inferior vena cava into a 2·5-ml syringe pretreated with 20 μl of 10% dipotassium EDTA. Collected blood was mixed well and blood cell numbers were determined with a Cell-DYN 3500 automatic counter (Dainabot Co., Ltd, Tokyo, Japan).

Isolation and flow cytometric analyses of platelets

Mice were bled from the inferior vena cava as described above. Pooled blood was centrifuged at 180 ***g*** for 10 min at room temperature to obtain platelet-rich plasma. Platelets were washed twice with a platelet washing solution (0·1 mM disodium EDTA and 0·05% NaN_3 in Dulbecco's PBS without divalent cations (D-PBS$^-$)) by centrifugation at 1200 ***g*** for 10 min.

To determine gating conditions for flow cytometric analyses of mouse platelets, freshly isolated platelets were mixed with 4 U/ml bovine thrombin (Nacalai Tesque, Kyoto, Japan) and incubated at room temperature for 5 min. Activated platelets were fixed with 2% paraformaldehyde at room temperature for 30 min, washed twice with the above washing solution, and resuspended in the washing solution containing 0·1% bovine serum albumin (BSA) at $1{\cdot}0\times10^6$ cells/10 μl. Fixed platelets were then incubated with FITC-conjugated anti-mouse CD62P MoAb (PharMingen, San Diego, CA) by adding 40 μl of the washing solution containing 0·1% BSA and 1 μg of the antibody to 10 μl of the platelet suspension. FITC-conjugated rat IgG1 (Caltag Labs, Burlingame, CA) was used as an isotype-matched control.

To detect platelet-associated IgG and IgM, MRL/+ and MRL/*lpr* mice were bled at 7 and 28 weeks old, and platelets were isolated as described above. Unfixed platelets were incubated with a mixture of FITC-conjugated goat anti-mouse IgG (γ-chain-specific; Cappel Organon Teknika) and PE-conjugated goat anti-mouse IgM (μ-chain-specific; Southern Biotechnology Associates, Inc., Birmingham, AL) antibodies as described above. Stained platelets were rinsed three times before being analysed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

For detection of retroviral gp70 antigenicities expressed in unfixed platelets, freshly isolated platelets were first incubated with rat anti-mouse CD16/CD32 antibody (PharMingen) added at 1 μg/10^6 platelets per 20 μl for 30 min at room temperature to block Fc receptors. Platelets were further incubated in the presence of the blocking antibody with a biotinylated anti-gp70 MoAb (either one of clones 24–8 (IgG2a [30]), 36D1.1 (IgG2a, this study) or 514 (IgM [32])) added at 10 μg/10^6 platelets per 30 μl. The methods for purification and biotinylation of MoAbs have been described [27,34]. After incubation at room temperature for 30 min platelets were rinsed twice with the washing solution and further incubated with PE-conjugated streptavidin (PharMingen) added at 0·5 μl/10^6 platelets per 50 μl, washed three times and analysed by flow cytometer. VL9G6 described above and an IgM MoAb, 9C12-1, specific for trinitrophenyl (TNP) hapten, which was produced and screened from immunized MRL/*lpr* mice by TNP-specific ELISA, were used as isotype-matched controls.

Immunoprecipitation and Western blotting

NZB-AR cells chronically infected with a biological clone of NZB xenotropic virus and control uninfected Mv1Lu cells were grown in 75-cm^2 tissue culture flasks to make confluent monolayers, and washed four times with ice-cold D-PBS. Mouse platelets were prepared and washed as described above. An extraction buffer (0·5% NP-40 in 50 mM Tris-buffered saline, pH 7·4, containing 10 mM disodium EDTA, 5 mM *n*-ethylmaleimide, 1 mM PMSF, and 0·002% leupeptin) was added at 1·5 ml per 75-cm^2 flask for cultured cells or 0·5 ml per 2×10^9 cells for platelets, and cells were lysed at 4°C for 15 min. The method for regular Western blotting has been described [22,27,34]. For immunoprecipitation, insoluble materials were removed by centrifugation at 15 000 ***g*** for 10 min and supernates were precleared by incubating with 15 μl/ml protein G-Sepharose 4FF (Amersham-Pharmacia Biotech, Uppsala, Sweden) at 4°C for 2 h with gentle agitation, followed by centrifugation at 1700 ***g*** for 5 min. Supernates containing cell extracts were next mixed with antibody-bound protein G-Sepharose that had been incubated with culture supernate of anti-gp70 antibody-producing hybridoma cells 36D1.1, and incubated overnight at 4°C with slow rotation. After washing, protein G-Sepharose with bound antigen was either subjected to Western blotting as follows or stored at 80°C till use.

For SDS–PAGE and Western blotting detection of precipitated antigens, the antigen-bound protein G-Sepharose was mixed with an equal volume of 4% SDS sample buffer [22,34] without a reducing reagent, and incubated for 15 min at room temperature. After centrifugation to remove the Sepharose beads, proteins in the supernates were separated through 7·5% polyacrylamide gel and transferred onto polyvinylidenedifluoride membrane (Immobilon; Millipore Corp., Bedford, MA) as described [22,34]. The blotted membrane was blocked with 10% skim milk, washed with D-PBS containing 0·05% Tween-20, and incubated with a 1:100 dilution of a biotinylated MoAb (either 36D1.1 or control VL9G6). Detection of bound antibody was done by chemiluminescence reaction using horseradish peroxidase-conjugated streptavidin (Zymed Labs Inc., South San Francisco, CA) and ECL + (Amersham Pharmacia Biotech) according to the manufacturers' instructions.

Transfer of hybridoma cells or purified antibodies into normal mice and pathological analyses

Hybridoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 4·5 g/*l* (final) glucose, 50 mg/*l* gentamycin sulphate, and 10% FCS, washed twice with PBBS, and resuspended in PBBS at approx. 10^7 cells/ml. (BALB/c × MRL/+)F_1 mice were transplanted intraperitoneally with $1–2\times10^7$ hybridoma cells after pretreatment with a 0·5-ml/mouse i.p. dose of 2, 6, 10, 14-tetramethylpentadecane (pristane; Aldrich Chemical Co., Inc., Tokyo, Japan) given 1–2 weeks prior to hybridoma transplantation. For purification of anti-gp70 and control IgG, hybridoma cells were grown in a serum-free medium (Hybridoma SFM; GIBCO BRL, Rockville, MD) using 4-*l* spinner flasks, and culture supernates were concentrated by using a tangential flow ultrafiltration system (Minitan II; Millipore Corp.). IgG was purified by Protein A-Sepharose (Amersham Pharmacia Biotech) affinity chromatography as described previously [27,34]. An indicated amount of purified MoAb dissolved in PBBS was injected into the tail vein after removing possibly contaminating immunoglobulin aggregates by centrifugation at 10 000 ***g*** for 15 min. The methods for preparation and staining of formalin-fixed, paraffin-embedded tissue sections and specimens for electron microscopy have been described [34].

Statistical analysis

Statistical analyses were done with the Prism software (GraphPad Software, Inc., San Diego, CA) using the most appropriate method of calculation recommended by the software.

RESULTS

*Development of thrombocytopenia and platelet-associated autoantibodies in MRL/*lpr *mice*

It has been shown that MRL/*lpr* mice spontaneously develop thrombocytopenia and their sera contain anti-cardiolipin autoantibodies [16,17]. We confirmed that platelet counts were lower in MRL/*lpr* mice ($(81 \pm 1{\cdot}7) \times 10^4/\mu$l (mean ± s.e.m.), $n = 5$) than in MRL/+ mice ($(103 \pm 2{\cdot}7) \times 10^4/\mu$l, $n = 5$) at 18 weeks in age ($P = 0{\cdot}0001$). To examine the possible presence of platelet-associated autoantibodies, MRL/*lpr* mice were bled at different ages and IgG and IgM antibodies on the surface of platelets were detected by flow cytometric analysis. The results demonstrated that circulating platelets in MRL/*lpr* mice at 7 and 28 weeks old were bound with a larger amount of IgG antibodies on their surface than those in MRL/+ mice (Fig. 1). In addition, the intensity of platelet-associated IgG in MRL/*lpr* mice was increased at 28 weeks in age in comparison with that at 5 weeks. These data support the notion that MRL/*lpr* mice do develop thrombocytopenia in association with platelet-associated antibodies.

Expression of endogenous xenotropic viral env *gene products and establishment from MRL/*lpr *mice and characterization of anti-gp70 MoAb*

To screen anti-gp70 autoantibody-producing hybridoma cells, the whole *env* gene isolated from an infectious molecular clone of NZB xenotropic virus, IU-6, was expressed in a vaccinia virus recombinant. Reactivities of a panel of anti-retrovirus MoAbs previously established from non-autoimmune mice [30–32] to the vaccinia virus-infected CV-1 cells confirmed the expression and proper antigenicity of this *env* gene product (N. Tabata *et al*., submitted for publication). Seven separate hybridoma clones were established from a fusion in which spleen and lymph node cells from four 2·5-month-old female MRL/*lpr* mice and NS-1 myeloma cells were used. These hybridoma cells were selected both for reactivity of secreted antibody with the CV-1 cells expressing the NZB xenotropic virus *env* gene and for the lack of reactivity to the cells infected with the control vaccinia virus–influenza virus HA recombinant. Western blotting and immunoprecipitation analyses demonstrated that MoAb 36D1.1 that was used in the following study precipitated and reacted with the whole *env* gene product, gp85 (gp70 + p15E), expressed in chronically infected NZB-AR cells (Fig. 2). This protein was not detectable from uninfected Mv1Lu mink cells, nor was it detectable with the control MoAb VL9G6, further confirming the specificity of this hybridoma antibody.

Pathogenicity of the anti-gp70 autoantibody

To explore possible nephritogenicity of the hybridoma-derived autoantibodies, each clone of the above anti-gp70 hybridoma cells was transplanted into pristane-treated syngeneic (BALB/c × MRL/+)F_1 mice, and the organs were examined histopathologically

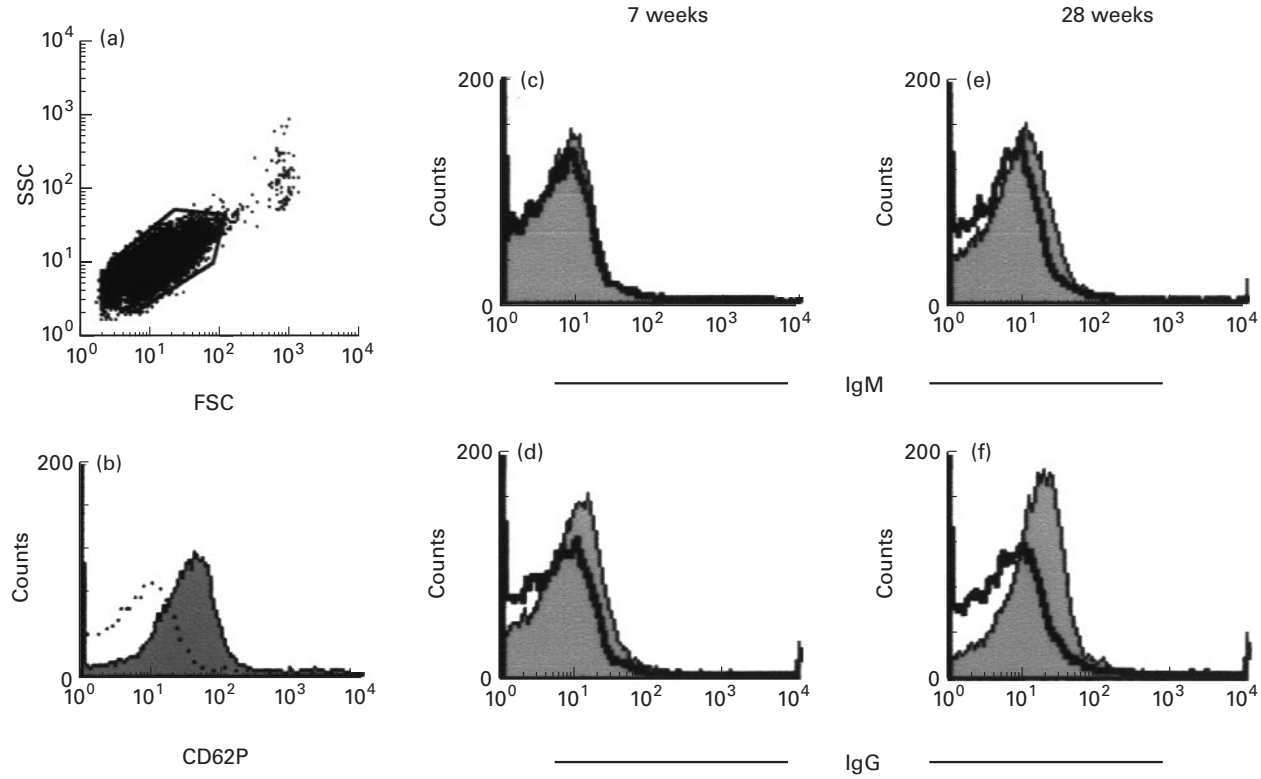

Fig. 1. Demonstration by flow cytometry of the presence of platelet-associated autoantibodies in MRL/*lpr* mice. (a,b) A typical gating condition adopted in the present study to analyse mouse platelets. FSC, Forward scatter; SSC, side scatter. When stained with anti-mouse CD62P MoAb after stimulation with thrombin, cells within the polygonal gate (a) showed a single peak (shadowed area in (b)) in flow cytometry analysis, confirming their homogeneity. (b) Dotted line shows the pattern of staining obtained when the same preparation of mouse platelets was incubated with the isotype-matched control antibody. (c–f) Detection of surface-bound IgM and IgG on mouse platelets. Platelets were isolated from MRL/+ and MRL/*lpr* mice at 7 and 28 weeks old. In each chart a thick solid line shows the staining pattern of MRL/+ mouse platelets, while a shadowed area indicates that of MRL/*lpr* mouse platelets.

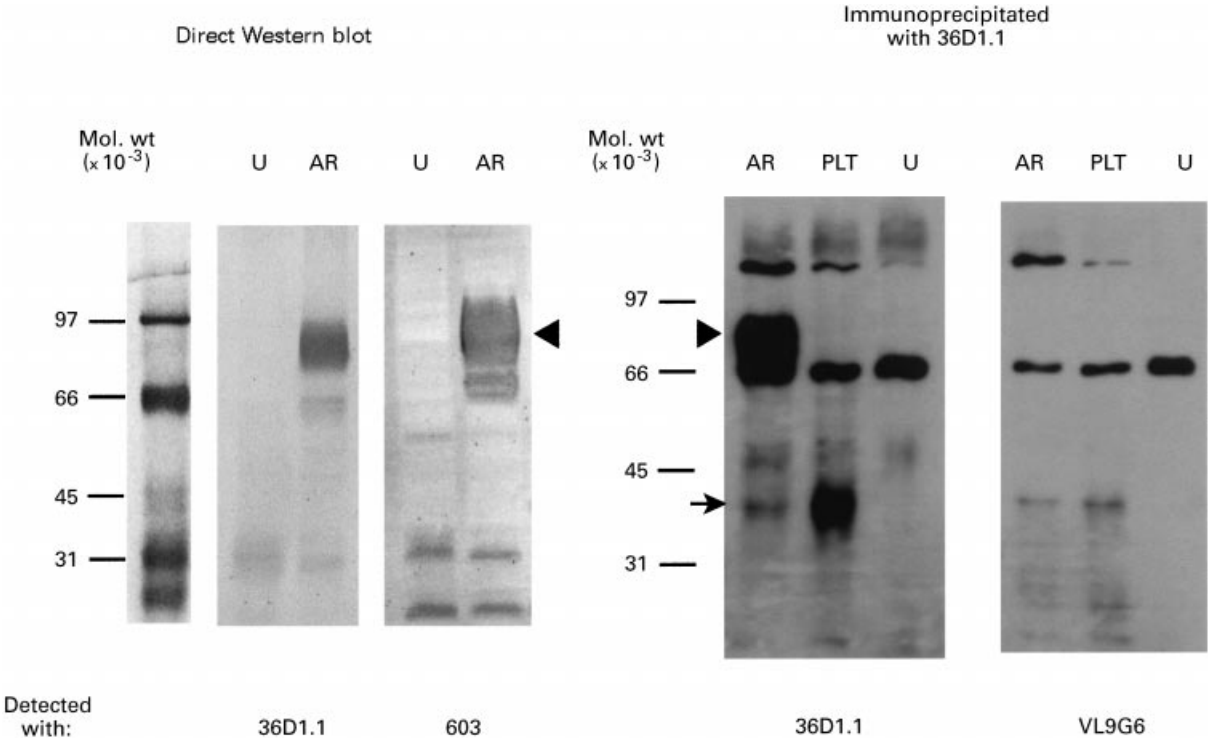

Fig. 2. Direct Western blotting and immunoprecipitation followed by Western blotting to detect the antigen reactive with the anti-gp70 MoAb. Extracts from uninfected Mv1Lu mink cells (U), NZB-AR cells chronically infected with an NZB xenotropic virus (AR), or (BALB/c × MRL/+)F_1 mouse platelets (PLT) were either directly subjected to SDS–PAGE (left) or immunoprecipitated with MoAb 36D1.1 before being separated by electrophoresis (right). Lanes mol.wt show positions of molecular weight markers with numbers on the left indicating relative molecular mass $\times 10^{-3}$ of each marker protein. Direct Western blotting confirmed the reactivity of MoAb 36D1.1 to retroviral gp85 (gp70 + p15E) that was also detected with the reference anti-gp70 MoAb, 603 [31]. The same protein was also precipitated from the extract of NZB-AR cells, but not from the extract of uninfected Mv1Lu cells, with MoAb 36D1.1 and detected with the same anti-gp70 MoAb (arrowhead). However, the precipitated gp85 was not detectable with the isotype-matched control antibody, VL9G6. A protein of approximately 40 000 in relative molecular mass was precipitated from mouse platelets and detected with MoAb 36D1.1 (arrow). Note that a faint band of apparently the same molecular mass was also detectable in the immunoprecipitate prepared from NZB-AR cells, but this band was absent from the blots of the extract prepared from uninfected Mv1Lu cells.

before the host mice died of tumour burden. During the course of this experiment, the F_1 mice transplanted with anti-gp70 antibody-producing hybridoma clone 36D1.1 were consistently found dead around 7 days after transplantation. Mice transplanted with any other clone of anti-gp70 antibody-producing hybridoma cells or control fusion partner cells did not die at this early stage after transplantation. Upon necropsy, bloody ascites associated with numerous foci of haemorrhage in the peritoneum and sometimes in the lungs were observed in mice transplanted with hybridoma 36D1.1. The above-described peritoneal haemorrhage was not observed in mice transplanted with other clones of anti-gp70 hybridomas.

To examine the causes of early haemorrhagic death, mice injected with hybridoma cells 36D1.1 were bled and killed daily after transplantation, their blood cells counted, and histopathologic and electron microscopic examination of their organs was performed. In comparison with the platelet numbers in the peripheral blood of mice transplanted with the control hybridoma, VL9G6, platelet counts were significantly reduced in (BALB/c × MRL/+)F_1 mice transplanted with anti-gp70 hybridoma 36D1.1 at 5 and 7 days after transplantation (Fig. 3). In addition, the number of erythrocytes was also significantly decreased at 5 and 7 days after transplantation of hybridoma 36D1.1, while such anaemia was not observed in the control mice. Histopathologic examination revealed disseminated microthrombi in capillaries and small arterioles of the lungs, which did not contain fibrin detectable with phosphotungstenic acid-haematoxylin staining (Fig. 4). Abnormal dilatation of glomerular capillary lumina associated with apparent segmental loss of mesangial matrix and occasional hyaline deposits were observed in the kidneys. Haemosiderin detected by Berlin blue staining was increased in the spleen of mice transplanted with 36D1.1 cells at 5 and 7 days after transplantation (Fig. 4), indicating the development of haemolysis. Electron microscopic examination of the pulmonary arterioles at 5 days after transplantation of hybridoma 36D1.1 revealed aggregated platelets in the vessel lumina (Fig. 5). Aggregates of platelets were also found in red pulps of the spleen (Fig. 5). These data show that platelets were aggregated *in vivo* both within the lumina of blood vessels and in the spleen of mice transplanted with hybridoma 36D1.1.

Induction of glomerular pathology by injecting purified anti-gp70MoAb

The above induction of thrombocytopenia by transplantation of the anti-gp70 autoantibody-producing hybridoma cells might be a

result of the tumour cell proliferation and invasion, or a consequence of possible production of a platelet-aggregating factor other than IgG. Therefore, we purified IgG from culture supernate of hybridoma 36D1.1, and syngeneic (BALB/c × MRL/+)F_1 mice were injected with the purified autoantibody. Mice were injected with either 12·5 μg or 50 μg/mouse of the purified antibody, and four mice as a group were killed and examined at each indicated time point along with the same number of control mice. The numbers of peripheral blood platelets and erythrocytes were not significantly different at 2, 3 and 7 days after antibody injection between the groups of mice injected with purified MoAb 36D1.1 and those injected with control IgG2a, although a few mice injected with MoAb 36D1.1 showed extremely low counts of platelets ($40{\cdot}2\times10^4/\mu$l, $44{\cdot}6\times10^4/\mu$l, and $69{\cdot}4\times10^4/\mu$l) at 2 and 3 days after antibody injection. However, when mice were killed at 7 days after MoAb injection and examined histopathologically, all eight mice injected with either 12·5 μg or 50 μg/mouse of the purified 36D1.1 MoAb showed a glomerular pathology that was not seen in the kidneys of control mice. The lesion was characterized by focal and segmental dilatation of capillary lumina with disappearance of mesangial matrix (Fig. 4). Hyaline thrombi were found in the lung of one mouse injected with purified MoAb 36D1.1 at 7 days after injection.

Expression of gp70 antigenicity on the surface of mouse platelets

To examine if the pathogenic anti-gp70 autoantibody directly bound to a platelet antigen, flow cytometry analyses of mouse platelets were performed. The presence of gp70 antigenicity on mouse platelet surfaces was demonstrated by significant staining of unfixed platelets with biotinylated anti-gp70 MoAb 36D1.1, as well as the biotin conjugates of two reference anti-gp70 MoAbs, 24-8 and 514 (Fig. 6). The isotype control MoAb of irrelevant specificity did not react with the same preparation of mouse platelets. Expression of gp70 antigenicity was not limited to (BALB/c × MRL/+)F_1 mice: in fact, mice of both the parent as well as MRL/*lpr* strains expressed the antigenicity detectable with MoAb 36D1.1 on the surface of their platelets (data not shown).

To identify the platelet proteins that were bound by the anti-gp70 autoantibody, combined immunoprecipitation and Western blotting was performed with the extract of mouse platelets. A distinct band of approximate molecular weight 40 000 was precipitated with the anti-gp70 autoantibody 36D1.1 and detected with the same anti-gp70 MoAb in Western blotting, but this protein was not detectable with the control IgG2a (Fig. 2).

DISCUSSION

MRL/*lpr* mice have been used as a model of human systemic autoimmune diseases, and pathogenic roles of autoantibodies reactive with endogenous retroviral gp70 in the development of lupus-like nephritis have been pointed out in several pathophysiological

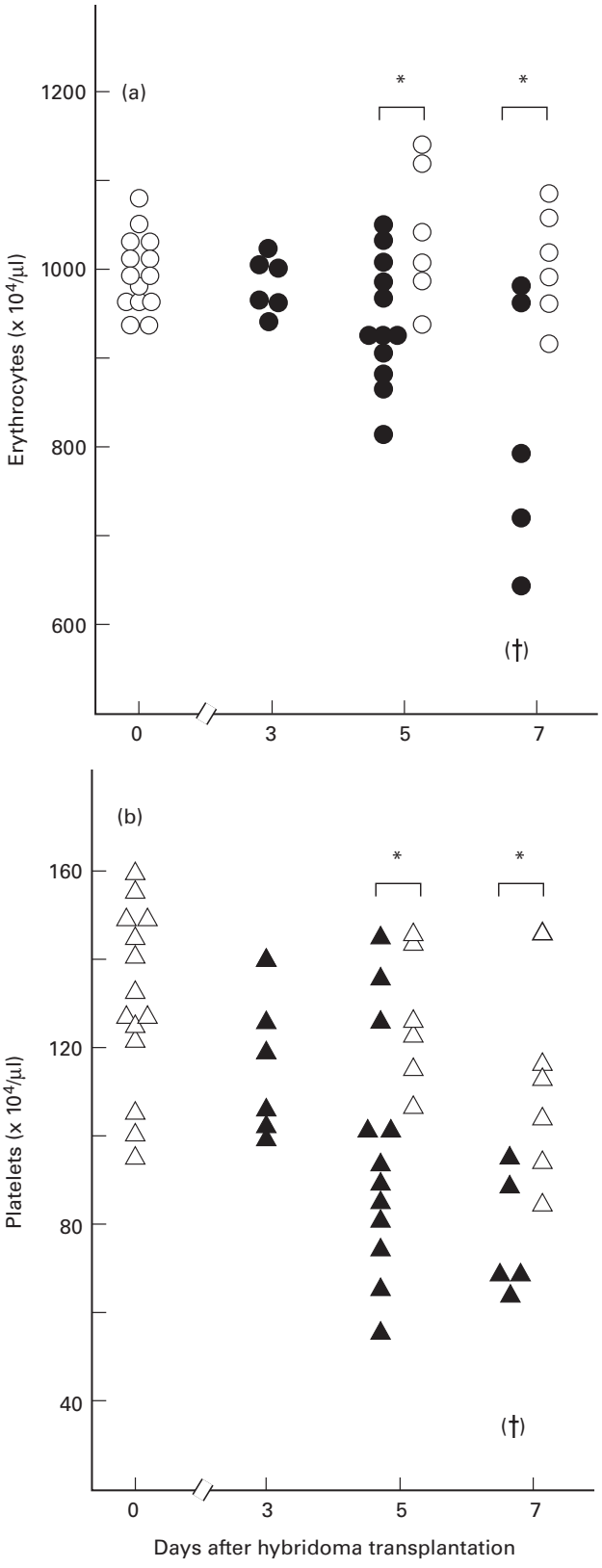

Fig. 3. Changes in the number of peripheral blood erythrocytes (a) and platelets (b) in (BALB/c × MRL/+)F_1 mice after transplantation with the anti-gp70 or control hybridoma cells. Each open symbol represents a blood cell number of an individual control mouse transplanted with hybridoma VL9G6, while closed symbols show those of the mice transplanted with anti-gp70 hybridoma 36D1.1. One mouse transplanted with 36D1.1 cells died before day 7 (†). *Significant difference between the indicated groups ($P < 0{\cdot}05$).

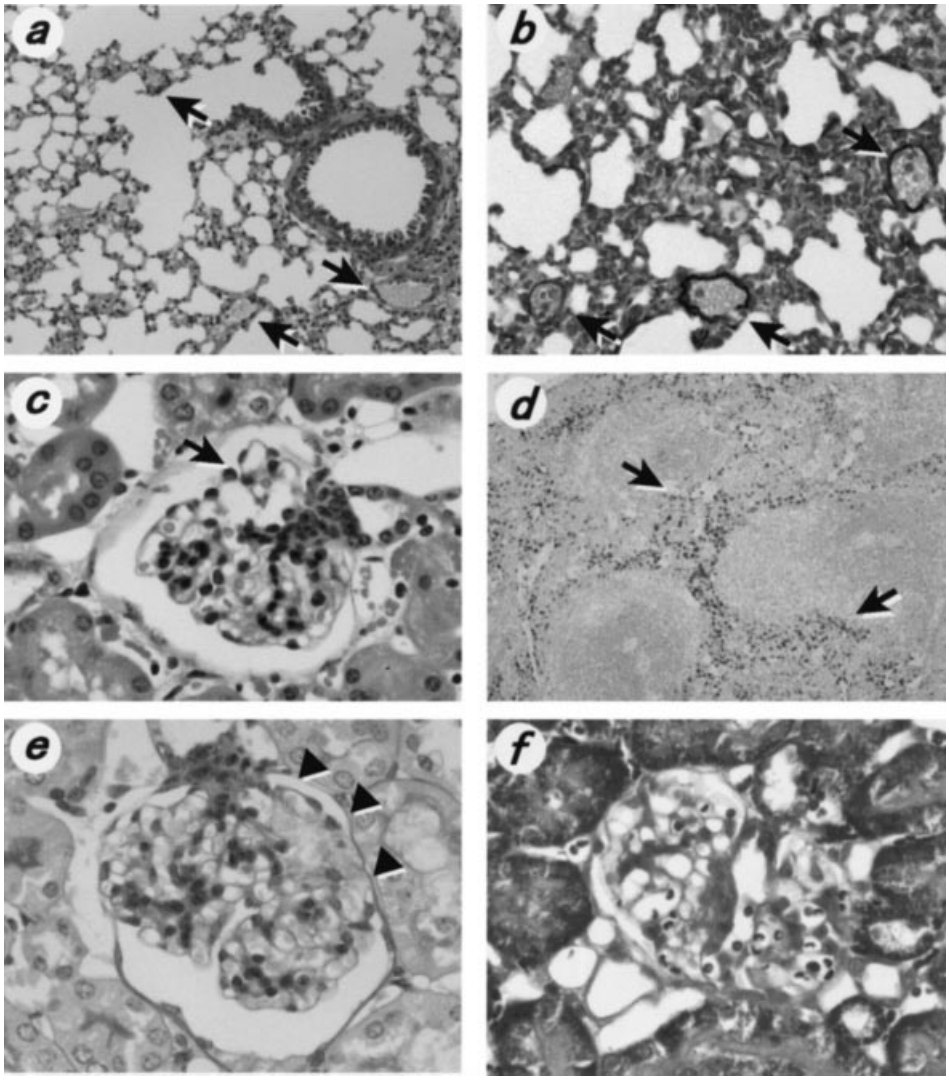

Fig. 4. Representative histopathology of mice transplanted with anti-gp70 hybridoma 36D1.1. (a) The lung with several sections of small arterioles (arrows) and venules that are filled with fine-granular platelet thrombi. (Haematoxylin and eosin, ×100.) (b) A representative section of the affected lung stained with phosphotungstenic acid-haematoxylin reagent to examine the presence of fibrin (×200). Fine-granular thrombi filling the arterioles (arrows) do not contain fibrin. (c) A representative section of the kidney. Note extreme dilatation of capillary lumina in the upper half (arrow), and segmental hyaline-like changes with disappearance of mesangial cells in the lower half. (Haematoxylin and eosin, ×260.) (d) Berlin blue staining of the spleen (×20) showing accumulation of haemosiderin in the red pulp (arrows) at 5 days after transplantation of hybridoma 36D1.1. (e,f) Representative glomerular pathology of mice injected with purified MoAb 36D1.1. Note the segmental hyaline-like change with disappearance of mesangial cells in (e) (arrowheads, periodic acid-Schiff stain, ×260). (f) Capillaries are abnormally dilated (Masson's trichrome stain, ×260).

and genetic studies of lupus-prone mice [18–20]. It has also been shown that MRL/*lpr* mice spontaneously develop thrombocytopenia and circulating anti-phospholipid autoantibodies [16,17]. However, the relationship between the production of various autoantibodies and the development of thrombocytopenia has not been elucidated. In the present study, we demonstrated the presence of platelet-associated IgG in MRL/*lpr* mice and the expression of the antigenicity of retroviral gp70 on the surface of mouse platelets. Furthermore, transfer of a single clone of anti-gp70 autoantibody-producing hybridoma cells into syngeneic non-autoimmune (BALB/c×MRL/+)F_1 mice resulted in platelet aggregation within blood vessels and thrombocytopenia followed by haemolytic anaemia. Injection of the purified anti-gp70 autoantibody into the same non-autoimmune mice consistently induced segmental dilatation of glomerular capillary lumina associated with disappearance of mesangial matrix. These pathological changes are reminiscent of glomerular lesions observed in an acute phase of human TTP that is referred to as glomerular paralysis [35].

Both MRL/*lpr* and (NZW×BXSB)F_1 models of lupus mice spontaneously develop thrombocytopenia in association with anti-platelet and anti-phospholipid autoantibodies ([13–17] and the present report). However, the exact causative relationship between these autoantibodies and the development of thrombocytopenia is not fully understood. Previous reports have characterized these mice as models of ITP or secondary anti-phospholipid antibody

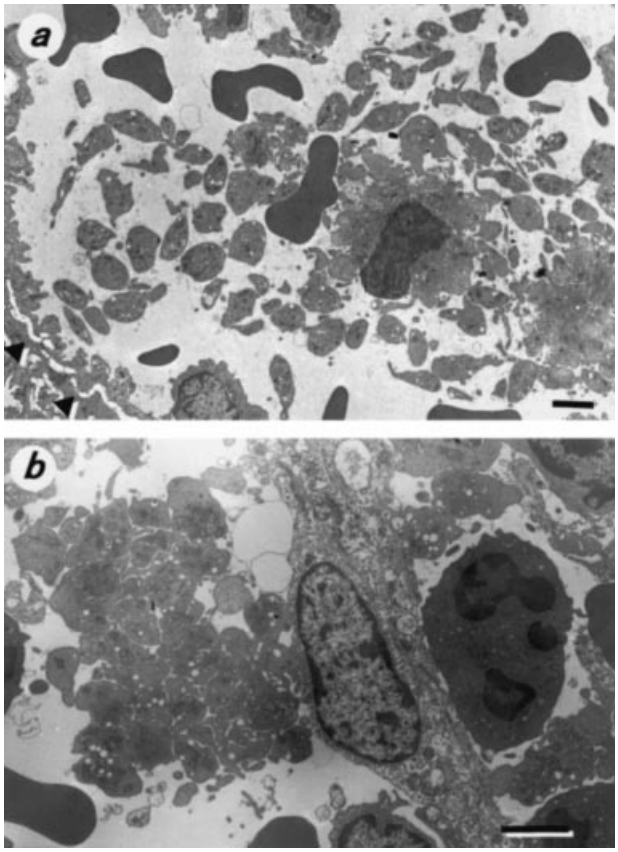

Fig. 5. Electron micrographs showing *in vivo* platelet aggregation in mice transplanted with hybridoma 36D1.1. (a) Aggregates of platelets within the lumen of a small arteriole in the lung. Arrowheads indicate elastic laminae. Note an erythrocyte is embroiled in an aggregate of platelets near the centre of the picture. Bar = 2 μm. (b) Aggregates of platelets in the splenic sinus. Bar = 2 μm.

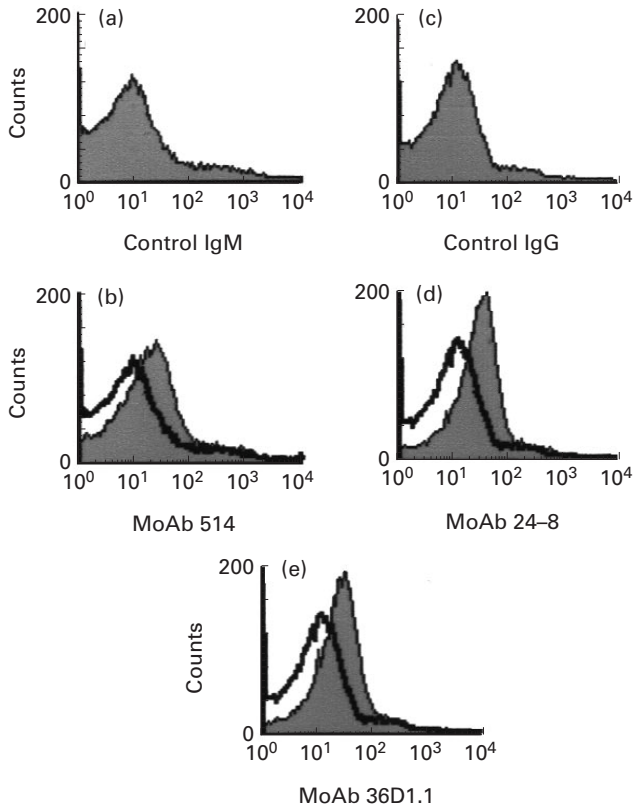

Fig. 6. The results of flow cytometric analyses demonstrating the expression of gp70 antigenicity on the surface of mouse platelets. (a,c) Staining patterns obtained with the control MoAb. These patterns are duplicated in (b,d,e) with a solid line for comparison. (b,d,e) Staining patterns obtained with anti-gp70 MoAb (shaded). Not only the pathogenic anti-gp70 MoAb 36D1.1 but two other anti-gp70 MoAbs of independent origins, 514 [29] and 24-8 [30], reacted to the surface of mouse platelets.

syndrome (APLAS) [13–17]. Although these two disease conditions and TTP are commonly characterized by thrombocytopenia, the underlying pathophysiology and clinical manifestations are quite different [10,11]. Thrombocytopenia is immune-mediated and due to premature sequestration of antibody-bound platelets by the reticuloendothelial system in the case of ITP and probably in APLAS. On the other hand, consumptive thrombocytopenia due to systemic thrombotic microangiopathy is a characteristic of TTP. As a result, microangiopathic haemolytic anaemia with the appearance in the peripheral blood of erthrocyte fragments always conjoins TTP, while immune-mediated haemolytic anaemia may or may not accompany ITP and APLAS. Of course, the pathophysiology of APLAS is more complicated because it accompanies recurrent thrombosis. However, thrombosis in APLAS affects arteries and larger veins, and microthrombosis, if present, is confined to the kidneys [10]. Thus, the formation of diffuse microvascular intraluminal platelet thrombi observed in mice transplanted with the anti-gp70 autoantibody-producing hybridoma cells as described in this study (Figs 4 and 5) is a hallmark of TTP, along with haemorrhagic manifestations, haemolytic anaemia (Fig. 3) associated with accumulation of haemosiderin in the spleen (Fig. 4), and glomerular paralysis which has also been induced with the purified autoantibody (Fig. 4). Since this anti-gp70 antibody-producing hybridoma clone was established by the fusion of spleen cells from unmanipulated MRL/*lpr* mice with non-producer myeloma cells, it may conceivably represent a part of a pathogenic autoantibody repertoire produced spontaneously in MRL/*lpr* mice. Therefore, at least a part of the pathogenesis of thrombocytopenia observed in these lupus-prone mice might be thrombotic microangiopathy, rather than immune-mediated sequestration. In this regard (NZW×BXSB)F_1 mice were originally reported to develop diffuse microthrombosis of the small branches of coronary arteries associated with focal infarction of myocardium [12]. This type of pathology is not usually encountered in ITP, while the heart is occasionally involved in TTP [2].

Animal models of TTP so far developed have utilized rats, dogs and pigs in which platelet aggregation was induced with vWF multimers that had been produced with a snake venom protein, botrocetin [36,37]. These models might be similar in their pathophysiology to the recently proposed mechanism of vWF-multimerization in acute episodes of human TTP [6,7], in which IgG autoantibodies to vWF-cleaving protease are shown to inhibit the enzyme activity, ultimately predisposing the patients to platelet thrombosis. However, TTP does have multiple aetiologies [2,3], and it is still unclear at present whether all cases of TTP can be explained by the unusual multimerization of vWF alone. In fact, a variety of systemic autoimmune diseases and immune abnormalities other than the protease-inhibiting antibodies has

been associated with this syndrome [4,5,8–11]. Nonetheless, until now no animal models of TTP were available in which possible roles of immunological mechanisms could be directly analysed. Our mouse model of hybridoma antibody-mediated microthrombosis might therefore be useful in elucidating the different roles of autoantibodies in induction of TTP, and may also contribute to the understanding of common pathogenic factors between TTP and SLE, which have long been suggested, but are still largely controversial [9–11].

Our pathogenic anti-gp70 autoantibody directly bound on the surface of mouse platelets. Antibodies reactive with platelets were also detected in the plasma of TTP patients [1–5,10]. The platelet protein precipitated and detected with the anti-gp70 autoantibody had an apparent molecular mass of 40 000. Thus, although three separate anti-gp70 antibodies of independent origins similarly reacted to mouse platelets, it is unlikely that the native form of retroviral *env* gene product, gp85 (gp70 + p15E), is present on the surface of mature platelets. In this regard, it has been shown that glycoproteins with approximate molecular masses of 45 000 and 32 000, known as gp45 and gp32, are readily detectable from purified mouse retroviral particles, and they are derived from the major glycoprotein gp70 by proteolytic cleavage [38]. Thus, the protein of approximate molecular mass 40 000 with gp70 antigenicity might represent a similar product of proteolytic cleavage derived from an endogenous counterpart of retroviral gp70. On the other hand, it might also represent a cross-reactivity of the anti-gp70 autoantibody to a platelet protein that is unrelated to retroviruses or *vice versa* (cross-reactivity of an anti-platelet autoantibody to gp70). Such cross-reactivity of anti-retrovirus antibodies to a platelet protein has been described in HIV-infected patients with ITP, in which the presence of a common epitope between HIV gp160 and platelet GPIIb/IIIa has been demonstrated [39].

Expression of an endogenous retrovirus in platelet-lineage cells, if that is the case, is not surprising. In fact, it is well known that an infection with exogenous Friend murine leukaemia retrovirus results in massive replication of this virus in megakaryocytes, associated with thrombocytopenia that becomes apparent within 7–9 days after infection [40]. In our previous study we demonstrated the expression of Friend virus *env* gene product in megakaryocytes at 10 days after the virus inoculation using a specific MoAb [27]. Thus, megakaryocytes may have an environment that supports effective expression of mouse retroviruses [40]. Furthermore, it has been shown that cells at particular stages of erythropoiesis in mice express *env* gene products of an endogenous retrovirus [41]. The expression of the endogenous retroviral gp70 on erythroid precursor cells varies from one strain to another, and the genetic locus that controls the expression of this *env* gene, *Rmcf*, is linked with resistance against leukaemia induced by infection with an exogenous retrovirus [42]. Therefore, it is similarly likely that in some strains of mice an endogenous retrovirus is expressed in platelet-lineage cells as a differentiation antigen.

Reactivity of the pathogenic anti-gp70 autoantibody with mouse platelet does not necessarily mean that the observed intravascular aggregation of platelets was a direct consequence of an antigen–antibody interaction. Rather, it is a widely accepted concept that binding of antibodies to specific epitopes on plasma membrane may induce activation and agglutination of platelets [10,43]. Thus, although the mechanisms of action of autoantibodies may vary from direct activation of platelets by binding onto their surfaces to abnormal multimerization of vWF through inhibition of the cleaving enzyme, the key event in the pathogenesis of TTP may be the same: formation of intraluminal platelet aggregation. Further studies are required to determine if and how the binding of the anti-gp70 autoantibody induces platelet activation.

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